EXHIBIT 8

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Consequences of a nectar yeast for pollinator preference and performance

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Summary

1. Pollinators utilize floral resources that vary in colour, scent and reward quality. Variation in such traits, including nectar rewards, in addition to cues associated with their quality, can influence pollinator foraging decisions with consequences for pollinator reproductive success. Nectar is commonly subject to colonization by micro-organisms capable of affecting a suite of traits important for pollinator attraction and fitness; yet, links between microbial presence and changes in pollinator preference and performance remain few.

2. Here, we evaluated the effects of a nectar-inhabiting micro-organism on pollinator foraging behaviour and reproduction using the common eastern bumblebee *Bombus impatiens* and the cosmopolitan nectar yeast *Metschnikowia reukaufii*. Using a combination of choice and no-choice behavioural and feeding assays, we manipulated the presence and viability of *M. reukaufii* in nectar and assessed bumblebee foraging and reproductive responses.

3. *Bombus impatiens* workers responded positively to the presence of yeasts. Foragers trained to associate yeast presence with flower colour visited a significantly greater proportion of flowers inoculated with yeast when subject to a colour discrimination test. Moreover, foragers naïve to nectar yeasts incorporated more yeast-inoculated flowers into initial foraging bouts when presented with a novel floral array. In addition, bees spent significantly longer foraging on yeast-inoculated flowers compared to yeast-free flowers. However, when we manipulated yeast presence and viability in microcolonies of queenless workers, we found no effect of yeast on components of bumblebee reproduction, such as initiation of egg laying and number of eggs laid. This lack of an effect of yeast persisted even under conditions of pollen limitation.

4. Taken together, these results suggest that nectar yeasts can enhance floral signalling and alter pollinator foraging behaviour at individual flowers, though they may not directly affect pollinator performance. Thus, nectar yeasts may play a significant role in mediating pollinator foraging behaviour, with consequences for plant fitness and evolution of floral traits.

Key-words: *Bombus impatiens*, *Metschnikowia reukaufii*, nectar yeasts, pollinator preference, pollinator reproduction

Introduction

Floral nectar is a critical resource that mediates plant–pollinator mutualisms. As the primary energy source for many pollinators such as bees, nectar not only fuels activity (Nieh *et al.* 2006), but can also be an important determinant of reproductive success (Pelletier & McNeil 2003). Nectar can also attract non-pollinating flower visitors, both seen and unseen, who exploit nectar for their own benefit at the potential expense of the plant and competing legitimate flower visitors (Adler & Bronstein 2004; Herrera, García & Pérez 2008; Irwin *et al.* 2010). Recent surveys have demonstrated that nectar-inhabiting micro-organisms (NIMs) are ubiquitous (Brysch-Herzberg 2004; Herrera, García & Pérez 2008; Fridman *et al.* 2012; Álvarez-Pérez & Herrera 2013) and are likely to interact with plants and pollinators. Yeasts, commonly present in nectar, can modify a variety of nectar traits important for pollinator attraction (Waller 1972; Alm *et al.* 1990; Dyer *et al.* 2006), including sugar

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content, pH, scent, amino acids and temperature (Raguso 2004; Herrera & Pozo 2010; Peay, Belisle & Fukami 2012; Vannette, Gauthier & Fukami 2013). These changes in nectar can have strong effects on plant fitness, ranging from positive to negative (Herrera, Pozo & Medrano 2013; Vannette, Gauthier & Fukami 2013; Schaeffer & Irwin 2014), mediated through changes in pollinator foraging behaviour. However, how NIMs affect the link between pollinator foraging preferences and pollinator fitness remains relatively unexplored, representing a critical missing link in our understanding of NIM effects on pollination mutualisms. Thus, while the presence of NIMs in nectar has been documented repeatedly, there is still little known about the consequences of these organisms for pollinator foraging decisions or whether the consumption of NIMs is beneficial or deleterious to nectar-feeding animals.

Optimal foraging theory predicts that pollinators should alter foraging behaviour in response to variation in nectar traits, which can be altered by NIM presence and metabolism (Emlen 1966; MacArthur & Pianka 1966). To forage optimally, pollinators such as bumblebees should avoid flowers colonized by NIMs, as NIM utilization of sugars can leave nectar less energetically rewarding (Herrera, García & Pérez 2008; Canto & Herrera 2012; Schaeffer, Vannette & Irwin 2015). Bumblebees are sensitive to changes in nectar traits, including sugar type (Pouvreau 1974; Mommaerts, Wäckers & Smagghe 2013), sugar concentration (Cnaani, Thomson & Papaj 2006) and volume (Real 1981, 1991). Alternatively, if NIMs ferment nectar (Wiens *et al.* 2008), flowers containing NIMs may remain attractive due to the caloric value of ethanol, which is nearly twice that of carbohydrates (Dudley 2000). Moreover, ethanol and volatiles associated with yeast fermentation may provide foraging cues to pollinators (Kevan *et al.* 1988; Ehlers & Olesen 1997; Raguso 2004), acting as an honest signal to indicate nectar presence and availability. One or more of these mechanisms may affect pollinator preference and behaviour.

A limited number of studies have tested the degree to which NIMs mediate pollinator foraging behaviour (Kevan *et al.* 1988; Herrera, Pozo & Medrano 2013; Vannette, Gauthier & Fukami 2013; Schaeffer & Irwin 2014). Both Herrera, Pozo & Medrano (2013) and Schaeffer & Irwin (2014) found that wild bumblebees remove more nectar from flowers densely populated with yeasts, suggesting a preference for flowers with NIMs. In addition, Herrera, Pozo & Medrano (2013) found that captive bumblebees (*Bombus terrestris*) respond positively to the presence of yeasts, visiting a significantly greater proportion of yeast-inoculated flowers. These effects may not be universal, however. Kevan *et al.* (1988) failed to detect an effect of yeasts on the foraging behaviour of honeybees. Moreover, Vannette, Gauthier & Fukami (2013) found that nectar yeasts did not affect the foraging behaviour of hummingbird pollinators, whereas nectar bacteria were a strong deterrent. Bacterial colonization of nectar can also have strong deterrent effects on honeybees and bumblebees (Good *et al.* 2014; Junker *et al.* 2014). Given these contradictory results, determining how NIMs affect pollinator decision-making may be pollinator- or context-dependent.

The effects of NIMs may not be limited to pollinator foraging behaviour, but also extend to changes in pollinator performance. Nectar comprises the primary energy source for many pollinators, such as bumblebees, hummingbirds and species of Lepidoptera (Proctor, Yeo & Lack 1996), and the abundance of floral resources available is an important determinant of performance and reproduction. For example, bumblebee colony development, worker production and/or reproductive success (production of new queens and males, hereafter referred to as sexuals) can be limited by floral resource availability (Bowers 1985; Sutcliffe & Plowright 1990; Pelletier & McNeil 2003; Williams, Regetz & Kremen 2012). Given the importance of floral resources for bumblebee development and reproduction, we still know surprisingly little about how variation in resource quality affects colony performance. Drastic reductions in sugar concentration (Herrera, García & Pérez 2008) and variation in amino acid content (Kevan *et al.* 1988; Peay, Belisle & Fukami 2012) in floral nectar through NIM activity may contribute to variation in nectar resource quality. NIM-induced changes in nectar sugar chemistry may lead to poor resource conditions, reducing colony production of workers and/or sexuals. Alternatively, NIMs, specifically yeasts, may enhance worker and sexual development due to their direct nutritional value as a protein and amino acid source (Northrop 1917). The degree to which costs and benefits of NIMs balance to affect pollinator performance has remained unexplored.

In a series of laboratory experiments, we tested the hypothesis that nectar yeasts affect pollinator preference and performance using the common eastern bumblebee *Bombus impatiens* Cresson (Apidae). We used two approaches to assess the potential for yeasts to mediate components of *B. impatiens* foraging behaviour. First, foraging worker bees were trained to associate a nectar treatment (i.e. yeast present or absent) with a floral trait (i.e. colour) and then subjected to a series of binary colour choice tests. Secondly, inexperienced (i.e. naïve) foragers were exposed to an array of novel flowers containing yeast-inoculated or sterile nectar to test whether bees could identify a cue associated with yeast presence and modify their foraging behaviour. Finally, we used a no-choice feeding assay to assess whether consumption of yeast and yeast-modified nectar affected the reproductive success of *B. impatiens* microcolonies. Together, these experiments provide a comprehensive test of the potential for a nectar micro-organism to affect the preference and performance of a common generalist pollinator.

Materials and methods

STUDY SYSTEM

We studied the behavioural and reproductive consequences of interactions between the bumblebee *B. impatiens* and the

nectar-inhabiting yeast *Metschnikowia reukaufii* (Metschnikowiaceae). As a generalist native forager, *B. impatiens* forages on a variety of plant species that regularly harbour nectar yeasts (Golonka & Vilgalys 2013), including *M. reukaufii*. For both the preference and performance experiments, we used commercial colonies of *B. impatiens* (Biobest Canada, Leamington, ON, Canada). *Metschnikowia reukaufii* is a cosmopolitan, ascomycetous yeast of the Metschnikowia clade. It is commonly associated with floral nectar and pollinators, with typical densities of 10^3–10^4 yeast cells mm^{-3} in floral nectar (Lachance *et al.* 2001; Herrera *et al.* 2009; Belisle, Peay & Fukami 2011; Schaeffer, Vannette & Irwin 2015). *Metschnikowia reukaufii* can degrade nectar, with total sugar concentration and per cent sucrose declining, and per cent fructose and glucose increasing, as yeast cell density increases (Schaeffer, Vannette & Irwin 2015). In our experiments, we used an isolated *M. reukaufii* strain (03-342o2, Marc-André Lachance, University of Western Ontario) collected from a milkweed beetle found on bindweed (*Calystegia sepium*) in Long Point, Ontario, Canada.

PREFERENCE EXPERIMENTS

We used commercial colonies of *B. impatiens* consisting of one queen and approximately 30–50 workers. Colonies were provided pollen *ad libitum* and, prior to experimentation, were connected to a screened flight cage (2·15 × 1·15 × 2·35 m) using a wire mesh tube with plastic gates. Outside of periods in which behavioural assays were conducted, colony workers were allowed full access to a foraging arena to collect 30% (w/w) sucrose solution from a multiwell feeder located in the flight cage. Workers making regular foraging trips between the colony and feeder were individually marked with paint pens for use in the behavioural assays (hereafter referred to as foragers).

Experiment 1: Preference test

Artificial flowers were constructed from 1·5-mL microcentrifuge tubes with either blue or yellow construction paper circles (diameter = 3·8 cm) attached around the mouth of the tubes, simulating artificial corollas. We used a blue–yellow colour dimorphism to facilitate easier discrimination on the part of the forager between the control and yeast nectar treatments (Gegear, Manson & Thomson 2007). We presented a pair of flowers to the foragers using a styrofoam board with flowers embedded upright and 10 cm apart. On a per-forager basis, each colour was assigned one of two treatments: 2 μL of either control solution [30% (w/w) sucrose solution] or yeast solution (*M. reukaufii*-inoculated 30% w/w sucrose solution inoculated 7 days prior to each replicate). The choice of a 7-day incubation period falls within the range of flower ages for species from which *M. reukaufii* has been isolated (Herrera, García & Pérez 2008). We trained marked bees to forage freely on arrays consisting of one flower type, with a training session consisting of successive foraging trips to each flower. This approach ensures that foragers experience each colour–nectar treatment combination before being subjected to the preference test. We randomized the colour–nectar treatment association across bees to control for potential colour bias in foraging decisions. Following training, foragers were presented with a paired array containing one flower of each treatment type and their foraging choice was recorded. When presented with a pair, once a choice was made, the other flower was covered and the pair was removed and replaced with a fresh pair of flowers. Each forager was presented with 10 paired arrays with flower colour location randomized for each array, and a proportion of choices to each colour was calculated for each individual bee. Once a bee had foraged on all 10 paired arrays, it was prevented from returning to the colony. This minimized the potential for yeast inoculation of honey pots within the colony, which could potentially inform foraging decisions of other workers. We tested a total of 22 workers from two source colonies (Colony 1: 13 bees; Colony 2: nine bees).

Statistical analyses. All analyses (here and below) were performed using R version 3.2.2 (R Core Team 2015). To assess whether yeasts affected foraging preference, we fit an intercept-only mixed model with individual bees and colonies as random factors to the proportion of visits to yeast-inoculated artificial flowers. This model was fit with a binomial error distribution using the LME4 package (Bates *et al.* 2015). A significant effect on preference was tested through examination of whether flower selection was non-random. This was achieved by comparing the intercept estimated in our model to an expected value under random foraging (0·5) using a two-tailed, one-sample *t*-test.

Experiment 2: Naïve forager test

Experiment 1 allowed us to test whether NIMs affected preference following exposure to flowers with vs. without yeast. Here, we focused on workers naïve to the nectar yeast treatment and floral phenotype (i.e. colour) to assess whether inexperienced bumblebees could respond to the presence of yeast. Naïve foragers were exposed to a novel, monochromatic flower array consisting of artificial flowers constructed as described in *Experiment 1*. We presented flowers to the foragers using a styrofoam board with artificial flowers ($N = 40$) embedded upright in a 4 × 10 grid, allowing foragers an equidistant choice between each flower in the array (distance between any two flowers: 8·5 cm). One of two nectar treatments were randomly assigned to flowers at each position: control (30% (w/w) sucrose solution) or yeast [*M. reukaufii*-inoculated 30% (w/w) sucrose solution inoculated 7 days prior to each replicate], respectively. We used a Sony Super SteadyShot HDR-SR11 high-speed video camera (Sony Electronics, San Diego, CA, USA) to record the number of visits to flower positions and foraging time per flower. Flowers were initially filled with 2 μL of solution and refilled using pipettes following each visit. Each bee ($N = 10$ workers from one colony) participated in only one assay and was prevented from returning to the colony. For each bee, new flowers were used, with nectar treatments randomized. When each bee finished foraging, bee size was estimated by measuring the length of the radial cell of the right wing, which correlates positively with bee size (Harder 1982).

Statistical analyses. To assess whether naïve foragers could respond to the presence of yeast, as in *Experiment 1*, we fit an intercept-only mixed model, with bee size as a covariate and bee identity as a random factor, to the proportion of visits to yeast-inoculated artificial flowers. A significant effect on preference was tested through examination of whether flower selection was non-random by comparing the intercept estimated in our model to an expected value under random foraging (0·5) using a two-tailed, one-sample *t*-test. To account for learning and the potential for preference to change as foragers gained experience, we also examined how the proportion of visits to yeast-treated flowers changed within a foraging bout. Visits were blocked by 5 for up to 35 flower visits (or seven blocks) and were compared using a one-way repeated-measures ANOVA with number of visits as a fixed factor and proportion of choices to yeast-treated flowers as a response (Gegear, Manson & Thomson 2007). Finally, to assess how nectar yeast treatment affected mean foraging time per nectar treatment, we used a linear mixed-effects model with nectar treatment as a fixed factor and bee size and identity as covariate and random effect, respectively. Foraging time was log-transformed prior to analysis to improve normality.

PERFORMANCE EXPERIMENT

We exploited the developmental strategy of queenless colony workers to assess the effects of nectar yeasts on the reproductive

success of *B. impatiens*. In queenless colonies comprised of multiple workers, one worker will establish dominance and act as 'queen' through development of oocytes, suppression of ovary development in subordinate workers, and laying male eggs (Cnaani, Schmid-Hempel & Schmidt 2002; Cnaani, Wong & Thomson 2007). We constructed microcolonies of queenless workers that were fed different diets to experimentally test the effects of yeasts on microcolony reproduction. Prior studies have documented that microcolonies provide good mimics of colony responses to changes in food resources (Tasei & Aupinel 2008). Workers for microcolonies were obtained from pupal clumps of commercial *B. impatiens* source colonies (Biobest Canada). Source colonies were fed 30% (w/w) sucrose solution *ad libitum*, and pollen was provided daily.

Treatments

We manipulated yeast presence (absent/present), viability (live/heat-killed) and pollen availability (low/high) in a fully crossed factorial design to assess the effects of yeast on pollinator performance and whether these effects varied depending on nutritional status. By manipulating yeast presence, viability and pollen availability, we could assess the contribution of yeast as a direct nutritional source for pollinators when pollen was in limited supply, as yeast can serve as a protein and amino acid source for other insects (Chippindale *et al.* 1993, 1997; Min & Tatar 2006). In addition, we could test for indirect effects of yeast on pollinator reproduction through potential changes in nectar chemistry due to yeast metabolic activity. We constructed 15 microcolonies per treatment combination, with each microcolony comprised of three worker bees obtained from one of seven source colonies. In cases where a worker perished during the experiment, the microcolony was terminated and discarded from future analyses, leaving 9–15 microcolonies per treatment.

Feeding microcolonies

Microcolonies were supplied with 3 mL of one of the following four nectar treatments daily. Sterile, 30% (w/w) sucrose solution served as the control. Our live yeast treatment consisted of the same solution inoculated with *M. reukaufii* (10^4 cells mm^{-3}) incubated at 25 °C for 4 days. A 4-day incubation period falls within the range of flower ages for plant species from which *M. reukaufii* has been isolated (Herrera, García & Pérez 2008). To ensure that yeasts had remained viable and active in solution in the live yeast treatment, we periodically plated subsamples on YM media and checked for colony growth. We also noted that the live yeast treatment had characteristic yeastlike odours indicative of fermentation, as well as increased turbidity, which is associated with yeast growth (R. Schaeffer, pers. obs.). To create the yeast-modified treatment, we followed the same procedure for creating the live yeast treatment; however, we removed yeast cells from the nectar via centrifugation (10 min at 4500 g) after the 4-day incubation period. Yeast cells were pelleted and the supernatant was removed and transferred to new sterile vials. Finally, to create the heat-killed yeast treatment, a 30% sucrose solution was inoculated with *M. reukaufii* at a density of 10^4 cells mm^{-3}. Immediately following inoculation, the solution was incubated at 90 °C for 15 min to kill cells. Pollen was supplied daily, consisting of a ball of pollen dough made with a 3 : 1 ratio of pollen to 30% sucrose solution. We used a wildflower pollen mix from Koppert Biological Systems (Howell, MI, USA). High- and low-pollen-availability (crossed with our nectar treatments) colonies received *c.* 300 mg or *c.* 30 mg balls, respectively. This reduction in pollen availability by an order of magnitude falls below the observed mean daily pollen consumption rate of a microcolony (57·6 ± 1·5 mg day^{-1}) (R. N. Schaeffer and R. E. Irwin, unpubl. data). Pollen balls and nectar feeders were weighed prior to and after microcolony feeding for 24 h, allowing us to obtain estimates of daily pollen and nectar consumption.

Fitness and viability

We monitored microcolonies daily to inspect for worker survival, egg laying, larval ejection and oophagy. Microcolonies were terminated 14 days post-egg laying (Tasei & Aupinel 2008). We then measured the following parameters of microcolony reproduction: (i) number of days to egg laying, (ii) number of larvae and eggs produced and (iii) weight of larvae and eggs. Measuring offspring production and quality (size) allowed us to assess protein assimilation and caloric intake (Simpson & Raubenheimer 1995; Pernal & Currie 2000; Manson & Thomson 2009).

Statistical analyses

We used separate linear mixed-effects models to assess the effect of dietary treatments on nectar and pollen feeding behaviour. Mean daily nectar and pollen consumption were calculated for each microcolony and used as response variables. Predictor variables included yeast presence, yeast viability, pollen availability and their interactions, mean colony radial cell length as a covariate, and colony of origin as a random effect. The interaction terms and covariate were tested for significance using Wald tests (ANOVA function in the CAR package), and then sequentially removed from the final models when not significant (Fox & Weisberg 2011). For *post hoc* pairwise comparisons between treatments, we applied Tukey's HSD tests of differences of least squares means.

To test for the effects of dietary treatments on the probability of egg laying, we used a generalized linear mixed-effects model (binomial error distribution) with source colony as a random effect. Mean radial cell length of the workers was included as a covariate, but was not significant and removed from the final model. For microcolonies that laid eggs, we used MANOVA to test how dietary treatments affected the number of offspring produced (eggs and larvae) and mean larval weight, with mean marginal cell length as a covariate. We used MANOVA to control for potential correlation between offspring production and larval weight because the same factors that may make colonies more productive in terms of egg laying may also make the larvae weigh more (Scheiner 1993). Because so few microcolonies in the low-pollen treatment laid eggs (see Results), in both models, we could not test for an interaction between dietary treatments and included main effects only.

Results

PREFERENCE EXPERIMENTS

Experiment 1: Preference test

Trained *B. impatiens* foragers preferred yeast-inoculated over yeast-free solutions. A significant proportion of choices made by trained individual foragers were for yeast-treated flowers (0·79 ± 0·06, mean ± SE), representing a significant departure from the expectation of random foraging (t = 5·07, d.f. = 21, P < 0·0001).

Experiment 2: Naïve forager test

Naïve foragers visited 35–67 flowers in a foraging bout. These foragers also responded positively to the presence of

yeasts in nectar. A significantly higher proportion of early visits to the flower array were to yeast-inoculated flowers (Fig. 1); however, this proportion varied significantly over time as foragers gained experience within a foraging bout ($F_{6,63} = 2{\cdot}59$, $P = 0{\cdot}03$). Across all flower visits in a bout, a higher proportion of visits were to yeast-treated flowers ($0{\cdot}60 \pm 0{\cdot}052$), though this did not significantly deviate from a pattern of random foraging ($t = 1{\cdot}92$, d.f. = 9, $P = 0{\cdot}09$). However, across all flower visits, foragers spent 34% longer foraging on yeast-inoculated flowers in comparison with controls ($F_{1,9} = 6{\cdot}39$, $P = 0{\cdot}03$).

PERFORMANCE EXPERIMENT

Pollen limitation affected some metrics of microcolony performance, but these impacts were not modified by nectar yeast treatments, nor did these diet treatments affect mortality ($\chi^2_3 = 1{\cdot}64$, $P = 0{\cdot}65$). The probability that microcolonies laid eggs was almost three times lower in the low- vs. high-pollen treatments (Fig. 2; $\chi^2_1 = 15{\cdot}62$, $P < 0{\cdot}0001$). In contrast, nectar yeast treatment had no significant effect on the probability of egg laying ($\chi^2_3 = 1{\cdot}01$, $P = 0{\cdot}80$). For microcolonies that produced eggs, we found no effect of the nectar yeast treatment (MANOVA: $\lambda = 0{\cdot}91$, $F_{6,38} = 0{\cdot}31$, $P = 0{\cdot}93$) or pollen treatment ($\lambda = 0{\cdot}91$, $F_{2,19} = 0{\cdot}97$, $P = 0{\cdot}40$) on the number of offspring produced or mean larval weight. Across all treatments, microcolonies produced $3{\cdot}96 \pm 0{\cdot}47$ offspring (range: 1–11 offspring) with mean larval weight of 14 ± 6 mg (range: 0·3–152·9 mg).

The effects on components of microcolony reproduction were reflective of changes in feeding behaviour in response to diet treatments. As predicted, pollen availability had a significant effect on pollen consumption. Microcolonies that were provisioned with a greater quantity of pollen consumed significantly more than those provisioned less (Fig. 2a; $\chi^2_1 = 77{\cdot}04$, $P < 0{\cdot}0001$). Moreover, we detected a significant effect of mean colony marginal cell length on pollen feeding ($\chi^2_1 = 10{\cdot}05$, $P = 0{\cdot}002$), indicating that microcolonies with larger bees consumed more pollen. In contrast, pollen consumption was not affected by any of the nectar yeast treatments ($\chi^2_3 = 4{\cdot}17$, $P = 0{\cdot}24$).

Pollen availability similarly had a significant effect on patterns of nectar consumption. Microcolonies in the high-pollen treatment consumed significantly more nectar than those provisioned with less pollen (Fig. 2b; $\chi^2_1 = 4{\cdot}44$, $P = 0{\cdot}035$). We detected a marginally significant effect of nectar treatment on nectar consumption ($\chi^2_3 = 6{\cdot}92$, $P = 0{\cdot}07$), with microcolonies exposed to the control nectar consuming upwards of 13% more nectar daily than the other treatments.

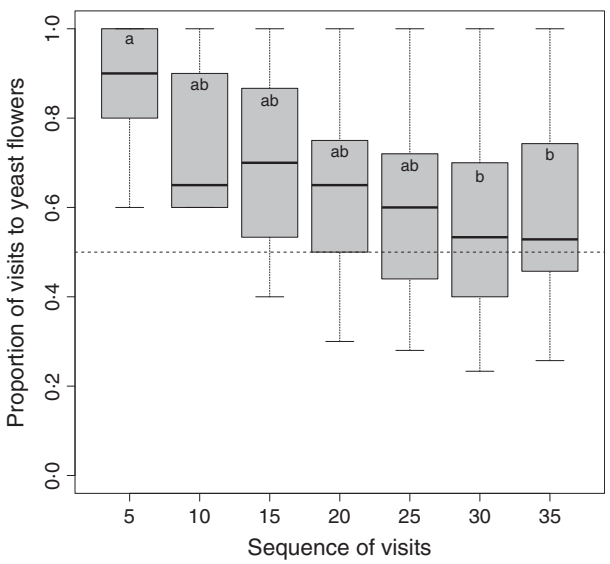

Fig. 1. Boxplots of the proportion of visits by naïve *Bombus impatiens* foragers to artificial flowers inoculated with the nectar yeast *Metschnikowia reukaufii*. Bars linked by the same letter are not significantly different as determined by a Tukey HSD test. The dashed line represents random foraging.

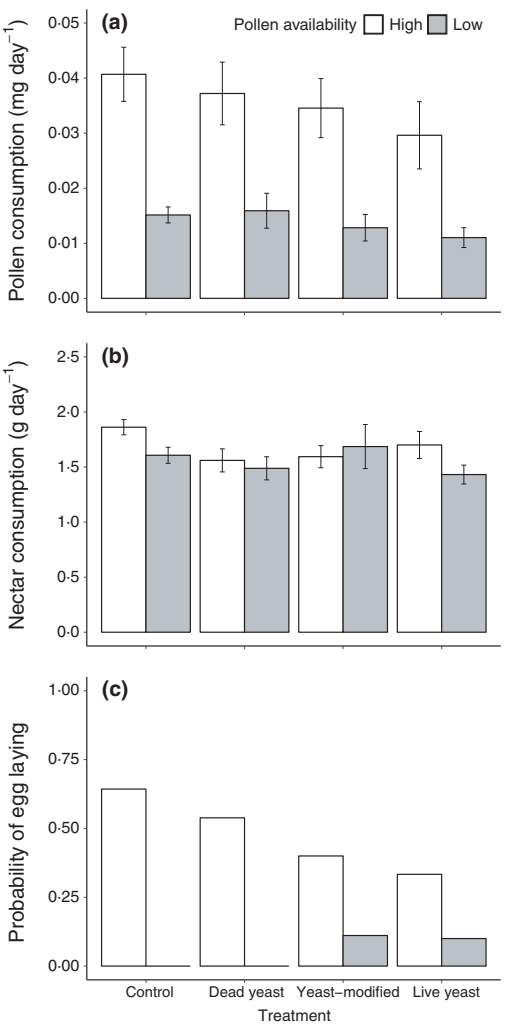

Fig. 2. Effects of pollen availability (high vs. low) and nectar quality (control, dead yeast, yeast-modified or live yeast) on (a) pollen consumption (mg day^{-1}), (b) nectar consumption (g day^{-1}) and (c) probability of egg laying for *Bombus impatiens* microcolonies.

Discussion

As ubiquitous members of plant–pollinator communities, NIMs have the potential to affect pollinator preference and performance through their presence and effects on an important energy resource, nectar. Conventional wisdom and predictions generated from optimal foraging theory (Emlen 1966; MacArthur & Pianka 1966) suggest that pollinators should be deterred by nectar yeasts, as yeast metabolic activity is capable of altering nectar sugar concentrations and ratios, making flowers less energetically rewarding (Pyke 1984; Heinrich 2004; Herrera, García & Pérez 2008). Our results are contrary to conventional wisdom, as *B. impatiens* foragers responded positively to yeast in both behaviour experiments. These findings, in combination with other recent studies (Herrera, Pozo & Medrano 2013; Vannette, Gauthier & Fukami 2013; Junker *et al.* 2014; Schaeffer & Irwin 2014; Schaeffer *et al.* 2014), highlight the significant role that NIMs may play in mediating pollinator behaviour, which, until recently, has largely been overlooked. Surprisingly, however, even though pollinators preferred flowers with yeast, neither yeast nor yeast metabolic activity within nectar affected microcolony performance, with nectar yeast treatments showing no benefits (or costs), even under pollen-limited conditions.

Though foragers responded positively to the presence of yeast, the proximate mechanisms driving changes in behaviour remain unknown. Bumblebees are capable of discriminating among flowers that differ in a variety of traits, including temperature, scent and nectar sugar concentration and composition (Kunze & Gumbert 2001; Cnaani, Thomson & Papaj 2006; Dyer *et al.* 2006; Mommaerts, Wäckers & Smagghe 2013). In *Experiment 1*, after learning to associate a particular nectar treatment with a flower colour, foragers were subjected to a series of flower colour choices. Given the high percentage of choices for yeast-treated flowers, this result suggests a preference for yeasts despite their potential effects on nectar sugar composition (Schaeffer, Vannette & Irwin 2015). Recent studies have similarly detected evidence of bumblebee preference for yeasts (Herrera, Pozo & Medrano 2013; Schaeffer & Irwin 2014; Schaeffer *et al.* 2014); in the field, wild bumblebees removed significantly more nectar from *Helleborus foetidus* and *Delphinium nuttallianum* flowers that contained yeasts in comparison with controls. Preference for yeasts by bumblebees could result from a number of mechanisms, including alteration of the taste profile through changes in sugar or amino acid composition (Herrera, García & Pérez 2008; Peay, Belisle & Fukami 2012). Moreover, the presence of vitamins, amino acids or metabolites such as ethanol may enhance the taste and attractiveness of yeast-inoculated flowers (Herrera, Pozo & Medrano 2013). Further research is needed to isolate the mechanisms contributing to changes in pollinator foraging behaviour.

Yeasts may also enhance the modality of floral signalling by providing an additional cue for the presence of nectar, which may affect the foraging behaviour of bumblebees. For example, if NIMs affect nectar scent (Raguso 2004), the presence of this additional cue may enhance colour discrimination by foragers (Kunze & Gumbert 2001). This may occur via improved attention towards visual cues if an olfactory signal associated with NIM presence and metabolic activity helps orient a forager. Alternatively, the combination of colour and scent may lead to better memory formation and retrieval (Kunze & Gumbert 2001; Kulahci, Dornhaus & Papaj 2008), which may explain the high level of accuracy of choices to yeast-inoculated flowers across foragers in *Experiment 1*.

The innate response of naïve foragers to the presence of yeast in *Experiment 2* provides support to the notion that bumblebees can identify a cue associated with yeast presence even before tasting the nectar. A greater proportion of initial visits by foragers with no previous training were to flowers containing yeast-inoculated nectar (Fig. 1). This suggests the potential for nectar yeasts to provide an honest signal of nectar presence, and resource availability, potentially through scent (Raguso 2004). However, as foragers gained experience and encountered more flowers, foraging decisions began to reflect random foraging as workers actively switched between yeast-inoculated flowers and control flowers, both of which contained an equal volume of sugar solution. This suggests that yeasts may be more important as a foraging cue rather than preferred resource and, moreover, that any potential benefits provided by yeasts do not outweigh the costs associated with bypassing other similarly coloured flowers once foragers learn that all are rewarding. Naïve *B. terrestris* workers responded in a similar manner to the presence of yeasts, as a significantly greater proportion of visits were to a feeder containing yeast-inoculated artificial nectar (Herrera, Pozo & Medrano 2013). Given variation in nectar availability in the field, pollinators may potentially rely on yeast olfactory cues to successfully identify resource patches. Beyond flowers and flower-visiting insects, other species such as *Drosophila* have been shown to rely on yeasts and yeast odours to aid in location of plant hosts for food and reproduction (Becher *et al.* 2012; Witzgall *et al.* 2012). While our use of artificial flowers in the studies above allowed us to isolate the effect of yeast on pollinator foraging decisions, the lack of odours produced by floral tissues may have accentuated our treatment differences. The role of yeast odours as a signal in mediating plant–pollinator interactions in natural systems therefore warrants further investigation.

Though we detected an effect of yeast on pollinator foraging behaviour, *M. reukaufii* is not the only yeast or micro-organism to colonize floral nectar (Brysch-Herzberg 2004; Álvarez-Pérez & Herrera 2013). Nectar yeasts and other nectar micro-organisms vary in a number of physiological traits (e.g. fermentation ability, osmotolerance), which may generate different pollinator responses depending on the identity of the micro-organism present. For example, although *B. terrestris* responded positively to the presence of yeasts in nectar, the magnitude of response

varied depending on the identity of yeast, as foragers responded more strongly to the presence of *M. reukaufii* as opposed to *M. gruesii* (Herrera, Pozo & Medrano 2013). Moreover, numerous pollinators have been shown to have a strong aversion to bacteria-colonized nectar, including bumblebees (Junker *et al.* 2014), honeybees (Good *et al.* 2014) and hummingbirds (Vannette, Gauthier & Fukami 2013). Deterrence is likely mediated by their effects on nectar traits, including reductions in nectar pH and alterations of both nectar sugar concentration and ratios (Vannette, Gauthier & Fukami 2013; Good *et al.* 2014). Future work is needed to elucidate how widely these results can be generalized and whether potential effects on behaviour are dependent upon micro-organism and pollinator identity, as well as the complexity of micro-organism communities in floral nectar.

Though pollinators responded positively to the presence of yeast, we cannot rule out the possibility that foraging on flowers inoculated with NIMs may still be maladaptive for bumblebees. NIMs may exploit pollinators to their benefit by manipulating their foraging behaviour to aid dispersal to new flowers (T. Fukami, pers. comm.). Results from *Experiment 1* may support such a notion. If NIMs affect floral signalling and enhance the accuracy of decisions made by foragers, this would promote floral constancy (Waser 1986), ensuring NIMs are vectored to hospitable, conspecific flowers. However, responding to a floral signal from NIMs may lead pollinators to make the energetically costly decision of visiting flowers with dense populations of NIMs, which often have lower concentrations of sugars (Herrera, García & Pérez 2008; Canto & Herrera 2012; Schaeffer, Vannette & Irwin 2015). Determining the potential costs of bypassing flowers that may be highly rewarding but lacking NIMs vs. the putative benefits of an honest signal of nectar from NIMs will likely depend on factors such as reward availability in the environment and forager experience.

Although bumblebees actively seek out yeasts in nectar, our results suggest that yeast consumption has no effect on bumblebee fitness. Even when we limited pollen availability, we still did not observe any fitness benefits or costs to yeast consumption. In our feeding trials, only pollen availability had a significant effect on a component of fitness measured (probability of egg laying), and the consumption of nectar yeast, a potential protein source, could not rescue egg laying under the experimental conditions of this laboratory study. Yeasts have been used as a protein supplement in honeybee colonies (Brodschneider & Crailsheim 2010) and can be an important source of amino acids or nitrogen for other invertebrates such as *Drosophila* spp. (Northrop 1917; Chippindale *et al.* 1993; Markow *et al.* 1999). Indeed, dry yeast (*Saccharomyces cerevisiae*) is rich in amino acids, which comprise *c.* 45% of its nutrient composition (Schulze 1995). Given that protein content of pollen can vary from 2·5% to 61% across plant species (Roulston & Cane 2000), yeasts may represent an important alternative protein source for pollinators when the amount of protein available in pollen is low. Our results suggest, however, that at ecologically relevant densities, yeasts do not affect bumblebee microcolony reproduction under experimental laboratory conditions. Thus, the direct effect of yeasts on bumblebees may be commensal, having no detectable fitness costs or benefits. This is not unlike many microbial gut associates found in bees that may derive a benefit from their host without imposing a cost or providing a benefit (Vásquez *et al.* 2012; Koch *et al.* 2013). However, it is important to note that even though there is no direct consumptive benefit (or cost) of consuming yeasts (or yeast-modified nectar), there is still the potential that yeasts may benefit pollinator energetics and performance in the field by providing an honest signal to the presence of nectar in flowers. Foraging bumblebees are faced with a patchy distribution of nectar rewards among individual flowers of the same and different flowering species. Yeasts may improve bumblebee nectar intake rates and energetics if they reduce the probability that bees visit nectar-free flowers. Given that nectar resource supplementation can increase bumblebee colony fitness (Pelletier & McNeil 2003), the degree to which yeasts enhance nectar intake in wild bumblebee colonies and the effects on colony fitness in the field require further investigation.

In conclusion, this study demonstrates that a NIM can mediate components of pollinator foraging behaviour. Future studies are needed that dissect not only the contribution of NIMs to landscape-level variation in nectar traits, but also how such changes impact pollinator foraging behaviour and pollinator fitness in the field. Finally, because pollinator foraging decisions can have strong effects on patterns of natural selection on floral and flowering traits (Galen 1989; Schemske & Bradshaw 1999), there is the potential that NIMs may indirectly affect pollinator-mediated selection. Considering the microbial context of plant–pollinator interactions may provide important insight into the evolutionary ecology of these interactions for flowering species.

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Data accessibility

Data deposited in the Dryad Digital Repository http://doi.org/10.5061/dryad.nn2h5 (Schaeffer *et al.* 2016).

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